Quantitative Model of the Enhancement of Peroxidase-Induced Luminol Luminescence

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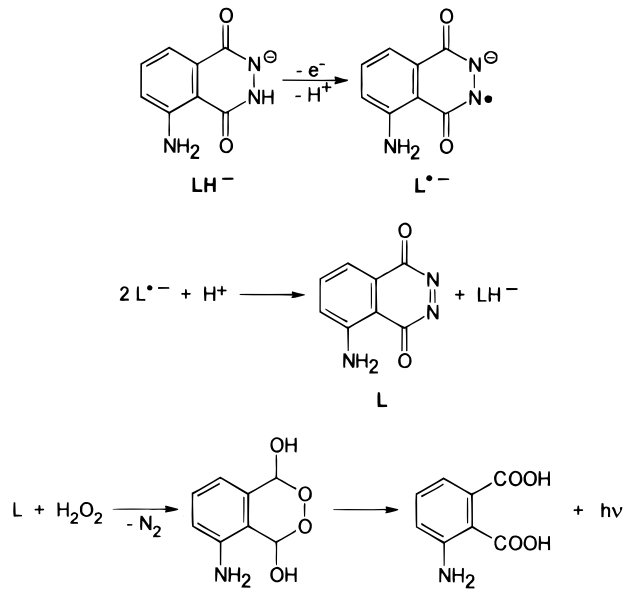
Abstract: Many phenolic compounds are known to enhance the chemiluminescence associated with the horseradish peroxidase-catalyzed oxidation of luminol, but the mechanism of enhancement is still unproved. Using stopped-flow spectrophotometry, we have found that a series of luminescence enhancers react rapidly with the peroxidase reactive intermediates (compound I and compound II) supporting the hypothesis that the enhancement is due to the acceleration of the enzyme turnover. In addition, pulse radiolysis experiments have shown that the enhancers' phenoxyl radicals oxidize luminol, consistent with a *redox mediator* role for the enhancers. The latter reaction was found to be reversible, showing that enhancers of low reduction potential, which are efficient in accelerating the enzyme turnover, are also scavengers of luminol radicals and therefore luminescence quenchers. Using these data, a simple model is proposed which correctly predicts that the efficiency of a phenolic compound as luminescence enhancer depends on the reduction potential of the respective phenoxyl radical according to a bell-shaped function with a maximum at ∼0.8 V.

Introduction

The oxidation of luminol (LH^-, 5-amino-2,3-dihydro-1,4-phthalazinedione) is accompanied by light emission. The well-established mechanism (Scheme 1) involves the formation of a luminol radical ($L^{\bullet -}$) that is further oxidized to a diazaquinone (L) which, on reaction with hydrogen peroxide, undergoes nitrogen elimination with ring opening to yield phthalate in an electronic excited state. The decay of the latter is responsible for the observed emission of light.[1−3] Peroxidases, such as the heme peroxidase from horseradish (HRP), can catalyze the oxidation of luminol, and this reaction is the basis of many chemiluminescent assays. Furthermore, it has been found that the intensity of the light emission can be augmented by the addition of the benzothiazole firefly luciferin.[4] Since then, several other luminescence enhancers have been discovered which have made chemiluminescence assays extremely sensitive.[5] However, the mechanism of the enhanced luminescence is poorly understood, and the discovery of new enhancers is usually achieved by trial and error.

The peroxidase-catalyzed oxidations involve the reaction of the native ferric enzyme (HRP) with a peroxide by oxygen transfer, formally a two-electron oxidation. The resulting compound I (HRP-I), which contains oxoiron(IV) coordinated to a porphyrin radical cation, returns to the ferric state by

Scheme 1



reaction with the reducing substrates (S) in two electron-transfer steps with compound II (HRP-II) as an intermediate, also an oxoiron compound but with no spin on the porphyrin. Each of these steps abstracts an electron from the substrate to form the respective radical cation ($S^{\bullet +}$):[6,7]

$$R-OOH + HRP \rightarrow HRP\text{-}I + R-OH \quad (1)$$

$$HRP\text{-}I + S \rightarrow HRP\text{-}II + S^{\bullet +} \quad (2)$$

$$HRP\text{-}II + S \rightarrow HRP + S^{\bullet +} \quad (3)$$

The reaction of luminol with compound I is ca. 100-fold faster

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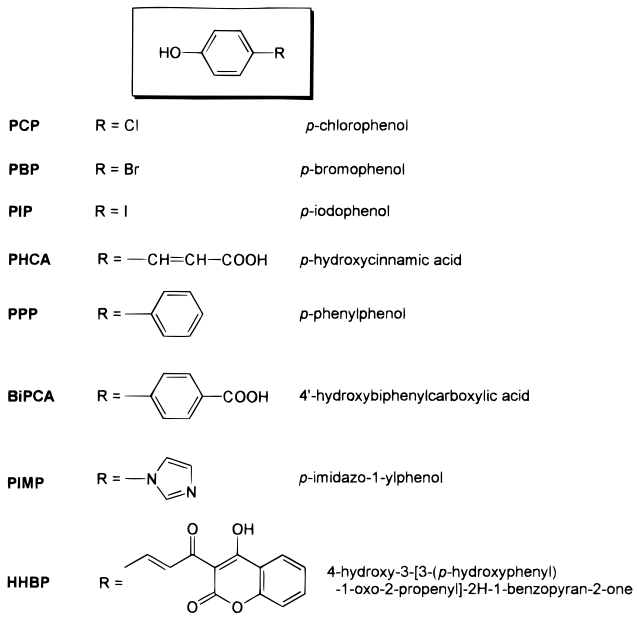

Figure 1. Structures and abbreviations of the phenolic compounds investigated in this study.

than the reaction with compound II.[8,9] In view of this kinetic behavior, it has been suggested that the enhancers act by reaction with compound II, thereby accelerating the turnover of the enzyme.[9,10] However, this does not fully explain the observed enhancement, and it has also been suggested that the enhancer may act as a redox mediator between the enzyme and luminol, i.e., it acts as the enzyme substrate and the resulting enhancer radical reacts with luminol in an electron-transfer reaction generating the luminol radical.[2,11]

In the present study, we have used pulse radiolysis to generate the radicals of a series of phenolic compounds that acted as chemiluminescence enhancers (Figure 1) and to monitor the electron-transfer reactions between those phenoxyl radicals and luminol. Those reactions were found to be reversible, and the corresponding equilibrium constants could be determined, which enabled the reduction potential of the phenoxyl radicals to be calculated. In addition, the rate constants of the peroxidase compounds I and II with the enhancers were measured by stopped-flow spectrophotometry. On the basis of the experimental results, a simple model of enhancement of chemiluminescence is proposed that enables a quantitative estimation of the efficiency of an enhancer on the basis of its redox properties.

Experimental Section

Horseradish peroxidase (HRP-4B) was purchased from Biozyme. 4-Hydroxy-3-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-2*H*-1-benzopyran-2-one (HHBP) was prepared according to the method of Oyama *et al.*[12] Other phenolic compounds were purchased from Aldrich or Sigma, and further chemicals and buffers were from BDH.

The determination of the chemiluminescence enhancement activity of the phenolic compounds was performed in an Amerlite plate reader from Amersham International, set at a dwell time of 0.2 s/well. The reaction mixture (90 μL) prepared in 0.1 mol dm^{-3} borate buffer at pH 8.5 contained sodium perborate (3 mmol dm^{-3}), luminol (0.5 mmol dm^{-3}), and enhancer (0.1 mmol dm^{-3}) added from a 10 or 100 mmol dm^{-3} stock in dimethyl sulfoxide. These solutions were placed in Microfluor "B" black, flat-bottom, 96-well microtiter plates (Dynatech), and reaction was initiated by the addition of 10 μL of a 5 nmol dm^{-3} solution of HRP.

The oxidation of luminol was also monitored spectrophotometrically, using a Hewlett-Packard 8452A diode-array spectrophotometer fitted with 1-cm quartz cells stirred magnetically. The reaction was carried out under the same conditions as the luminescence experiments, except for the peroxidase concentration, which was 15 times higher to compensate for the inferior sensitivity of this detection method, relative to the luminometry. The increase of absorbance at 520 nm was monitored taking readings at 1 s intervals for up to 100 s. The initial rate was determined by fitting a straight line to the initial points.

The rates of reaction of peroxidase compounds I and II with the luminescence enhancers were determined using a stopped-flow spectrophotometer, Model 1705 from Applied Photophysics, by the method described previously.[9] In brief, solutions of horseradish peroxidase compound I were prepared by mixing native (ferric) enzyme (2 μmol dm^{-3}) and hydrogen peroxide (2 mmol dm^{-3}) in Tris-HCl buffer (0.05 mol dm^{-3}) and NaCl (0.1 mol dm^{-3}) at pH 8.5. These solutions were rapidly mixed in the reaction cell with solutions of the phenolic compound (20−400 μmol dm^{-3}), and the formation of compound II via reaction 2 was monitored by the buildup of the absorbance of compound II at 426 nm, the isobestic point of the spectra of ferric enzyme and compound I. The concentration of the peroxidase was chosen to give an easily detectable absorption, and the excess of phenolic compound assured pseudo-first-order conditions and completion of the reaction within the time scale accessible to the stopped-flow spectrophotometer. The observed rate of formation of compound II was proportional to the concentration of the phenolic compound, and the rate constant for reaction 2 ($k_{\text{cpd-I}}$) was determined from the slope of the linear plots of observed rate against concentration.

For the determination of the rate of reaction of compound II with the phenolic compounds, similar experiments were performed but with the concentration of hydrogen peroxide only 2 μmol dm^{-3} in the reaction mixture. Upon mixture with the phenolic compound (10−200 μmol dm^{-3} in the reaction mixture), compound II was formed, and its subsequent decay was monitored by the absorbance at 426 nm. This rate was also proportional to the concentration of the phenolic compound, and the $k_{\text{cpd-II}}$ was determined from the slope of the linear regression.

Pulse radiolysis was performed with a 4 MeV van de Graaff accelerator as described previously.[13] Pulses of 10 ns were used which delivered doses of ca. 1 Gy, as determined by thiocyanate dosimetry.[14] The solutions were prepared in 10 mmol dm^{-3} phosphate buffer at pH 8.5 and contained 0.05 mol dm^{-3} sodium azide and variable concentrations (up to 1 mmol dm^{-3}) of luminol and/or the phenolic compound. Before irradiation, these solutions were saturated with oxygen-free nitrous oxide (from British Oxygen Company). All radiolysis experiments were performed at room temperature (22 ± 2 °C). Under these conditions, the pulse of electrons generates the azidyl radical ($N_3^{\bullet}$) in less than 1 μs and with a radiation chemical yield of ca. 0.6 μmol J^{-1} (refs 15 and 16).

Results

(i) Enhancement of Chemiluminescence. The phenolic compounds investigated in the present study increased the intensity of the chemiluminescence resulting from the HRP-catalyzed oxidation of luminol. The enhancement efficiency (*f*) was measured by the maximum intensity relative to the system without enhancer, as listed in Table 1.

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Table 1. Maximum Intensity of Peroxidase-Induced Luminol Luminescence in the Presence of Enhancers

enhancer	f_{obs}[a]
PIMP	1435 ± 176
BiPCA	968 ± 83
PPP	922 ± 117
PIP	642 ± 104
HHBP	367 ± 46
PHCA	214 ± 25
PBP	186 ± 19
PCP	132 ± 24

[a] Relative to value in the absence of enhancer ± standard deviation of the mean.

Table 2. Rates of Reaction of Horseradish Peroxidase Compounds I and II with Enhancers in Aqueous Solution[a] at pH 8.5 and Room Temperature

compd	$k_{cpd\text{-}I}$/dm^3 mol^{-1} s^{-1}	$k_{cpd\text{-}II}$/dm^3 mol^{-1} s^{-1}
luminol[b]	8.0×10^5	1.2×10^4
PIMP	1.2×10^6	1.8×10^5
BiPCA	1.9×10^6	3.8×10^5
PPP	3.9×10^7	2.6×10^6
PIP[b]	2.8×10^7	3.4×10^6
HHBP	5.0×10^6	8.3×10^5
PHCA	6.0×10^7	1.9×10^7
PBP[b]	8.3×10^6	5.7×10^5
PCP[b]	2.4×10^6	3.6×10^4

[a] In 0.05 mol dm^{-3} Tris and 0.1 mol dm^{-3} NaCl. [b] Values from ref 9.

The oxidation of luminol leads to colored products with an absorption maximum at 520 nm. Spectrophotometric experiments showed that the phenolic compounds accelerated the formation of these products. The initial rate of increase of absorbance at 520 nm observed in the presence of each of the enhancers was proportional to the maximum luminescence intensity (results not shown).

(ii) Reaction of Enhancers with HRP Compounds I and II. The rates of reaction of peroxidase compounds I and II with the luminescence enhancers were measured by stopped-flow techniques. The obtained values are listed in Table 2. All the compounds reacted faster with compound I than with compound II ($k_{cpd\text{-}I} > k_{cpd\text{-}II}$) as is typical of peroxidase reactions. The rates were also considerably higher than those obtained previously with luminol under the same conditions.[9]

(iii) Reaction of Phenoxyl Radicals with Luminol. Pulse radiolysis experiments with azide solutions containing the phenolic compounds showed the formation of transient species with absorption in the visible range. This absorption decayed on a time scale of milliseconds by second-order kinetics and with first half-life proportional to the radiation dose. From these results, we conclude that the azide radical reacted with the phenolic compounds to generate the respective radicals. Previously, $N_3^\bullet$ has been shown to react with phenols (Ph−OH) to yield phenoxyl radicals (Ph−O$^\bullet$), and our results are in agreement with that conclusion.[15,17]

$$N_3^\bullet + \text{Ph}-\text{OH} \rightarrow N_3^- + \text{Ph-O}^\bullet + H^+ \qquad (4)$$

The observed second-order decay of absorption shows that the phenoxyl radicals are decaying by bimolecular reactions, and the rate of these reactions could be determined from the linear dependence of the reciprocal of the first half-life on the initial radical concentration, i.e., on the absorbed dose (Table 3).

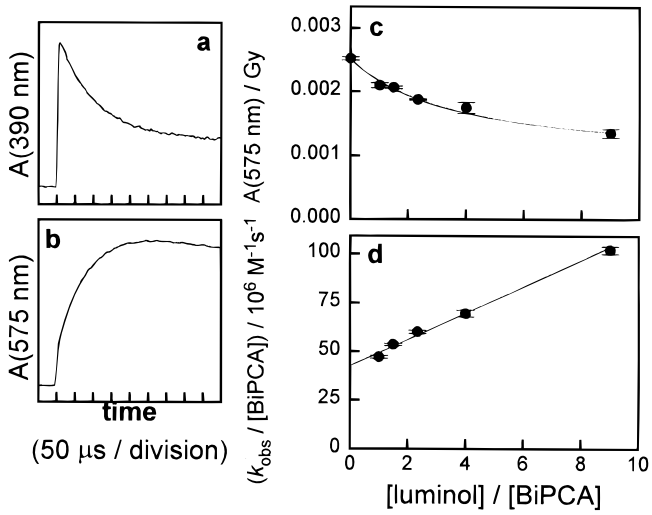

Figure 2. Electron-transfer equilibrium between BiPCA phenoxyl radicals and luminol anion at pH 8.5. The transient absorbance shows the fast formation and slow decay of luminol radical anion (a, 390 nm) and the slow formation of the BiPCA phenoxyl radical (b, 575 nm) in a solution containing 0.8 mmol dm^{-3} luminol and 0.2 mmol dm^{-3} BiPCA. The absorbance at equilibrium (c, 575 nm) and the rate of approach to equilibrium (d, 575 nm) depended on the [luminol]/[BiPCA] ratio, from which the equilibrium constant of electron transfer was calculated.

Table 3. Spectral Properties and Rates of Bimolecular Decay of the Radicals of Luminescence Enhancers at pH 8.5 and Room Temperature

compd	λ_{max}/nm	ϵ/dm^3 mol^{-1} cm^{-1}	$2k$/dm^3 mol^{-1} s^{-1}
PIMP	410	4120	1.5×10^9
BiPCA	575	4370	9.4×10^8
PPP	560	4360	1.9×10^9
PIP	450	3150	3.2×10^9
HHBP	635	2110	1.6×10^9
PHCA	595	2040	8.0×10^8

$$2\text{Ph}-\text{O}^\bullet \rightarrow \text{products} \qquad (5)$$

Previous studies by Merényi *et al.*[18,19] have shown that the azidyl radical reacts also with the luminol anion (LH^-) to give the respective anion radical ($L^{\bullet -}$), with an absorption maximum at 390 nm:

$$N_3^\bullet + LH^- \rightarrow N_3^- + L^{\bullet -} + H^+ \qquad (6)$$

We have performed pulse radiolysis experiments with azide solutions containing mixtures of luminol and phenolic compounds. Immediately after the pulse, an increase of absorption was observed, both at 390 nm and at the wavelengths of maximum absorption of the phenoxyl radicals. This observation is consistent with a competition between luminol and the phenolic compounds for the $N_3^\bullet$ formed by irradiation, resulting in a mixture of luminol and phenoxyl radicals. On the time scale of 10^{-4} s, it was observed that the absorption at 390 nm decayed whereas the absorption at the λ_{max} of the phenoxyl radical increased further (Figure 2a,b). Both transformations followed exponential curves and had rates equal within the experimental uncertainty. The absorption measured at the time of completion of these transformations varied with the concentrations of luminol anion and phenolic compound.

(17) Lind, J.; Shen, X.; Eriksen, T. E.; Merényi, G. *J. Am. Chem. Soc.* **1990**, *112*, 479−482.

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These observations show that the luminol radical anion reacted with the phenolic compounds to yield the respective phenoxyl radical:

$$L^{\bullet -} + Ph{-}OH \rightleftarrows LH^{-} + Ph{-}O^{\bullet} \quad (7)$$

The apparent rate of this reaction (k_{obs}) depended upon the concentrations of both phenolic compound and luminol anion. Such behavior can be explained by the reversibility of reaction 7, in which case the integration of the appropriate differential equations,[20] with the approximation of negligible radical−radical reactions, leads to a k_{obs} dependent on the concentrations of both compounds:

$$k_{obs} = k_f[Ph{-}OH] + k_r[LH^{-}] \quad (8)$$

where k_f and k_r are the rate constants of the direct and reverse reaction 7. In agreement with this hypothesis, plots of k_{obs}/[Ph−OH] against [LH^-]/[Ph−OH] gave good straight lines (Figure 2d), which allowed the determination of k_f and k_r from the slope and intercept, respectively. The values thus determined were used to estimate the equilibrium constant of reaction 7, $K_{kin} = k_f/k_r$ (Table 4).

It can also be demonstrated[20] that the absorbance at equilibrium (A_{eq}) obeys the expression

$$A_{eq}/C_R = (\epsilon_{PhO} + \epsilon_L K[LH^{-}]/[Ph{-}OH])/(1 + K[LH^{-}]/[Ph{-}OH]) \quad (9)$$

where C_R is the total concentration of radicals at the radiation dose used, ϵ_{PhO} and ϵ_L are the extinction coefficients of the phenoxyl and luminol radicals at the wavelength of the measurement, and K is the equilibrium constant of reaction 7. Plots of A_{eq} against [LH^-]/[Ph−OH] were constructed and the equilibrium constants (K_{abs}) determined by nonlinear least squares fit of eq 9 to the data (Figure 2c). The equilibrium constants determined by both methods (K_{kin} and K_{abs}) are listed in Table 4.

Discussion

The luminescence measurements show that all the phenolic compounds investigated in this study enhance the peroxidase-induced luminol chemiluminescence (Table 1), and on the basis of absorbance measurements, this could be correlated to an increase in the rate of oxidation of luminol. From this observation, it could be concluded that the chemiluminescence enhancement is caused by the acceleration of the chemical process leading to the emitting species (excited phthalic acid) and does not involve any significant effect on the physical process of light emission. This is in agreement with previous conclusions[5] based on the similarity of the emission spectra observed in the absence and presence of enhancers.

In his pioneering study Cormier[8] found that, in the absence of enhancer, the intensity of emitted light is proportional to the square of the rate of generation of luminol radicals. The quadratic relation is a consequence of the mechanism of generation of the excited species, which involves the dismutation of two luminol radicals:[1]

$$2L^{\bullet -} + H^{+} \rightarrow LH^{-} + L \quad (10)$$

In the presence of enhancer, reactions 5 and 11 may also take place:

(20) Wardman, P. *J. Phys. Chem. Ref. Data* **1989**, *18*, 1637−1755.

Table 4. Kinetic and Equilibrium Constants for the Electron Transfer between Luminol and Enhancer Radicals in Aqueous Solution at pH 8.5 and 22 ± 2 °C

compd	λ/nm	$k_f/10^6$ dm^3 mol^{-1} s^{-1}	$k_r/10^6$ dm^3 mol^{-1} s^{-1}	K_{kin}	K_{abs}
PIMP	500	nd[a]	nd[a]	nd[a]	0.662 ± 0.024
BiPCA	390	3.6 ± 0.2	36 ± 1	0.100 ± 0.006	0.090 ± 0.019
	575	7.3 ± 0.5	42 ± 3	0.174 ± 0.017	0.285 ± 0.046
PPP	560	1.0 ± 0.6	110 ± 10	0.0093 ± 0.055	0.136 ± 0.012
HHBP	635	6.7 ± 0.5	180 ± 20	0.038 ± 0.005	0.065 ± 0.011
PHCA	390	3.4 ± 0.2	48 ± 1	0.070 ± 0.004	0.047 ± 0.008
	595	4.6 ± 0.5	59 ± 2	0.078 ± 0.009	0.033 ± 0.051

[a] Absorbance change too small to determine rate of establishment of equilibrium.

$$Ph{-}O^{\bullet} + L^{\bullet -} \rightarrow \text{products} \quad (11)$$

Both the rate of reaction 5 for each of the enhancers and the rate of reaction 10 have been determined by pulse radiolysis (Table 3).[1] They exhibit little variation, with values all close to $\sim 10^9$ dm^3 mol^{-1} s^{-1}, as is typical of reactions between radicals. The rates of reaction between the phenoxyl radicals and the luminol radical (eq 11) could not be determined. However, these reactions being also reactions between two organic radicals, it is likely that these rates will have similar values of $\sim 10^9$ dm^3 mol^{-1} s^{-1}. Therefore, it can be assumed that radical−radical reactions do not perturb significantly the electron-transfer equilibrium (eq 7), and thus an approximate relation between the luminescence intensity and the square of the rate of generation of luminol radicals should hold also when the luminescence is enhanced by phenolic compounds. In this case, the luminescence intensity can be expected to depend on (a) the rate of enzyme turnover and (b) the electron transfer between enhancer radicals and luminol. In the following, we will demonstrate that the detailed characterization of both factors leads to a quantitative description of the enhancement process.

The pulse radiolysis results demonstrate the reversibility of the oxidation of luminol by the phenoxyl radical (eq 7), in agreement with previous observations with one of the enhancers studied (*p*-iodophenol).[19] The reversibility of this reaction is of fundamental importance to the enhancement mechanism: if the enhancer acts as a redox mediator between the peroxidase and luminol, the oxidation of the latter may not be complete, and therefore the intensity of light emission may be below the value that would be achieved if that oxidation were irreversible. In effect, on the basis of our results it can be concluded that, upon oxidation of a phenolic compound in the presence of luminol, a redox equilibrium will be established. The constants of equilibrium 7 listed in Table 4 are <1, which implies that, even for equal concentrations of luminol and enhancer, the formation of enhancer radicals at the cost of luminol radicals will be thermodynamically favored. Under the conditions of the pulse radiolysis experiments, which are typical of those of the luminescence measurements, the redox equilibrium was established in less than 1 ms; for longer times or under conditions of steady-state generation of radicals, the equilibrium can be kinetically shifted by the decay of the species involved, i.e., of the luminol or enhancer radicals. However, as discussed above, the rates of bimolecular decay of the radicals investigated are not dissimilar ($k \sim 10^9$ dm^3 mol^{-1} s^{-1}), and therefore this reaction is not expected to have a pronounced effect on the redox equilibrium.

Thermodynamically, the position of equilibrium 7 is determined by the difference between the reduction potentials of the phenoxyl and luminol radicals under the conditions of the experiment, as given by the Nernst equation:

Table 5. Reduction Potentials[a] of the Radicals of Luminol and Enhancers in Aqueous Solution at pH 8.5 and 22 ± 2 °C

compd	$E_{8.5}$/V vs NHE[a]	compd	$E_{8.5}$/V vs NHE[a]
luminol	0.82[b]	HHBP	0.74
PIMP	0.81	PHCA	0.75
BiPCA	0.76	PBP	0.87[c]
PPP	0.73	PCP	0.85[c]
PIP	0.87[c]		

[a] Weighted averages of the K_{abs} and K_{kin} values listed in Table 4 were used. [b] Based on the value in ref 19 and corrected for pH. [c] Calculated from the value in ref 17.

$$E_{8.5}(\mathrm{Ph{-}O^{\bullet},H^{+}/Ph{-}OH}) - E_{8.5}(\mathrm{L^{\bullet -},H^{+}/LH^{-}}) = -(RT/F)\ln K \quad (12)$$

where T is the absolute temperature and R and F are the gas and Faraday constants. The reduction potential of the luminol radical, as well as its acid−base properties, has been investigated previously,[19] and the results allow the calculation of the reduction potential at pH 8.5. Here we have used this value to calculate the reduction potentials of the enhancer radicals on the basis of the experimentally determined equilibrium constants (Table 5).

The reduction potentials of the enhancer radicals may be expected to govern not only the equilibrium constant of the redox mediation but also the rate of reaction of the enhancers with the peroxidase reactive intermediates. In effect, previous studies by Dunford *et al.*[21,22] have demonstrated that the rates of reaction of horseradish peroxidase compounds I and II with a series of phenols and anilines depend on the substituents in a systematic fashion, i.e., electron-donating substituents strongly increased the rate of the reaction. Linear relations between log k and the Hammett substituent parameters (σ) were found, which was taken as evidence for electron transfer as the rate-determining step in the oxidation of phenols. At the time of these studies, reduction potentials of the phenoxyl radicals were not available, but those of some para-substituted phenoxyl radicals were determined later using pulse radiolysis[17] and found to depend on the the substituents, with a linear dependence on the Brown−Okamoto substituent parameters (σ^{+}). The correlation of $k_{\text{cpd-II}}$ and reduction potentials with different substituent parameter scales has raised some concern.[23] We found that the $k_{\text{cpd-II}}$ values determined by Dunford and Adeniran[22] are linearly dependent on the reduction potentials of the respective phenoxyl radicals (correlation coefficient −0.91):

$$\log k_{\text{cpd-II}} = \alpha - \beta E_{7.6} \quad (13)$$

The values of the constants α and β were obtained from the least-squares fit:

$$\alpha = 9.4 \pm 0.7 \quad \text{and} \quad \beta = 4.7 \pm 0.9\ \mathrm{V^{-1}} \quad (14)$$

Unfortunately, the only phenols for which the reduction potentials of the respective radicals are known react with horseradish compound I with diffusion-limited rates. For this reason, a relation of the type shown in eq 13 for $k_{\text{cpd-I}}$ cannot be confirmed.

We have plotted the rates of reaction of the enhancers with compounds I and II determined by stopped-flow techniques against the reduction potentials determined by pulse radiolysis (Figure 3). A large scatter is seen, not surprisingly if the disparate structure of the compounds is considered. For the rates of reaction with compound II, the relation expressed by eq 13 is shown for comparison. Interestingly, it does not deviate considerably from the linear regression through the enhancers' data, suggesting that despite structural effects the rate of reaction of compound II with phenols is determined largely by the reduction potential of the phenoxyl radical.

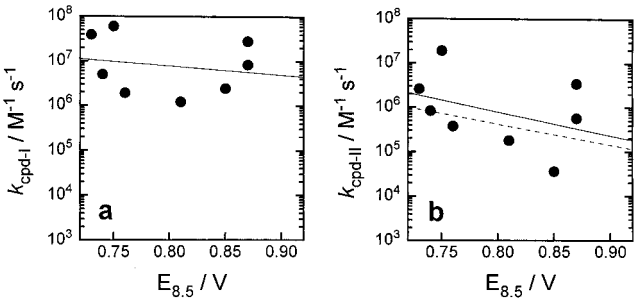

Figure 3. Rates of reaction of phenolic compounds with horseradish peroxidase compound I (a) and compound II (b) at pH 8.5 as a function of the reduction potential of the respective phenoxyl radicals under the same conditions. The solid lines are the linear least-squares fits; the dashed line in panel b is based on the rates determined by Dunford *et al.*[21,22] and the reduction potentials determined by Lind *et al.*[17]

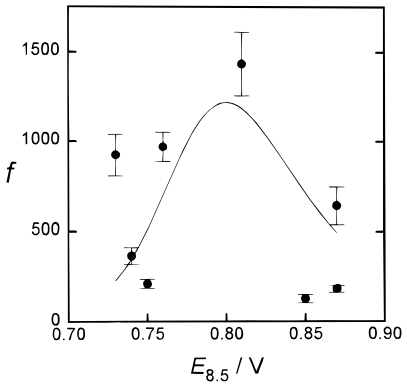

Figure 4. Relative enhancement efficiency of the phenolic compounds (f) as a function of the reduction potentials of the respective phenoxyl radicals. The solid line is the curve calculated according to the model discussed in the text.

Equations 12 and 13 show the opposing effects of the oxidizability of phenolic compounds on their efficiency as enhancers of chemiluminescence. Compounds for which the respective phenoxyl radical has a high reduction potential are not easily oxidized and can be predicted to be ineffective in accelerating the rate of peroxidase turnover. On the other hand the corresponding constant of equilibrium 7 is high, and therefore these compounds are expected to be good redox mediators. Reciprocally, compounds that form phenoxyl radicals of low reduction potential are easily oxidized and efficiently accelerate the turnover of the enzyme, but they are poor redox mediators as the equilibrium 7 will be shifted to the right and they may even quench the luminol radicals.

Figure 4 illustrates this concept, showing a bell-shaped dependence of the luminescence enhancement efficiency on the reduction potential of the enhancer radicals. Furthermore, the maximum luminescence enhancement is obtained with the compound for which the reduction potential of the phenoxyl radical is close to that of the luminol radical (*p*-imidazol-1-ylphenol, PIMP). These ideas can be quantified assuming the proportionality between luminescence intensity (f) and square of the rate of generation of luminol radicals (r_{L}), as discussed above,

(21) Job, D.; Dunford, H. B. *Eur. J. Biochem.* **1976**, *66*, 607−614.
(22) Dunford, H. B.; Adeniran, A. J. *Arch. Biochem. Biophys.* **1986**, *251*, 536−542.
(23) Colclough, N.; Smith, J. R. L. *J. Chem. Soc., Perkin Trans. 2* **1994**, 1139−1149.

$$f = Ar_L^2 \quad (15)$$

where A is the proportionality constant. We propose that the rate of generation of luminol radicals is given by the rate of enzyme turnover (r_{TO}), weighted by the fraction of the radicals generated that result in luminol radicals ($L^{\bullet -}$) after redox mediation equilibrium:

$$r_L = r_{TO}/[([Ph{-}OH]/[LH^-])K + 1] \quad (16)$$

The rate of enzyme turnover is governed by the rate-determining step, the reduction of compound II to ferric enzyme:

$$r_{TO} = 2C_{HRP}(k_{cpd\text{-}II\text{-}L}[LH^-] + k_{cpd\text{-}II}[Ph{-}OH]) \quad (17)$$

Equation 13 was used to calculate $k_{cpd\text{-}II}$ as a function of $E_{8.5}$, using the α and β values derived for the enhancer data (α = 10.2; β = 5.3 V^{-1}). The resulting values, together with the equilibrium constants calculated by eq 12, were inserted into eqs 15 and 17. Finally, the arbitrary proportionality factor A was adjusted by nonlinear least-squares fit to the experimental data; the resulting line is shown in Figure 4. It is remarkable that this model, which does not contain adjustable parameters other than the vertical scaling factor, reproduces the bell-shape dependence of the luminescence enhancement on the reduction potential as well as the position of the maximum at $E_{8.5} \sim 0.8$ V.

In conclusion, the enhancement of chemiluminescence can be described quantitatively and to a good approximation by considering (a) the acceleration of the enzyme turnover by reaction of the enhancer with compound II and (b) the *reversible* electron-transfer reaction between the enhancer radical and luminol. On the basis of this model, it is predicted that the most efficient luminescence enhancers are compounds whose radicals have reduction potentials of the order of ~0.8 V.

Although the proposed model does explain the overall features of the enhancement intensity, important variations are observed for individual compounds. In the model, the assumption was made of a universal relation between the rate of reaction of compound II with any phenolic compound and the reduction potential of the respective radical. The coarseness of this approximation is evident in Figure 4. Hodgson and Jones[10] have suggested that the most efficient enhancers are those compounds for which the rate of reaction with peroxidase compound II is higher than predicted on the basis of Hammett relations. This is equivalent to accounting for the discrepancies between the observed enhancer efficiencies and the model proposed here on the basis of $k_{cpd\text{-}II}$ only. However, the data in Figures 3 and 4 do not support this hypothesis. Other factors, such as the stability of the enhancer radicals or rapid enzyme inactivation, must have a small but non-negligible effect on the enhancement efficiency.

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